THE RIBOSOMAL S4-RNA FRAGMENT MELTS COOPERATIVELY WHEN COMPLEXED WITH PROTEIN S4

David G. BEAR*, Thomas SCHLEICH, Harry F. NOLLER, Steve DOUTHWAITE+ and Roger A. GARRETT+

Division of Natural Sciences, University of California, Santa Cruz, CA 95064, USA and +*Max-Planck-Institut für Molekulare Genetik, Ihnestr. 63–73, D-1000-Berlin-Dahlem 33, Germany*

Received 25 October 1978
Revised version received 17 January 1979

1. Introduction

Ribosomal protein S4 is a key protein for the in vitro assembly of the 30 S subunit of *Escherichia coli* (e.g., [1]). It binds to a large region at the 5′-end of 16 S RNA, defined as the S4-RNA [2–4], which can be prepared in large quantities by digestion of either renatured 16 S RNA or a protein S4–16 S RNA complex with carrier-bound pancreatic ribonuclease [4,5]. The S4-RNA binds exclusively protein S4 [2,4] and is the first cognate protein-binding site to be isolated from the 16 S RNA in large amounts.

Previously, the topography of the RNA, in both the free S4-RNA and the protein-complexed form (S4–RNP), was compared by two approaches.

(1) The location and % yield of each carrier-bound ribonuclease cut and sequence excision was approximately determined. It was found that whereas the positions of the cuts were the same in the S4-RNA and S4–RNP, the yields of the cuts were higher by a small but significant amount in the S4–RNP [3].

(2) The S4-RNA and S4–RNP were reacted with kethoxal such that accessible non-base paired guanines were modified [6]. Most of the modification sites were the same in the absence and presence of the protein, but the reactivity of a few sites increased significantly in the presence of protein S4. Both of these experiments suggested that some opening of the S4-RNA structure occurred as a result of protein binding.

With a view to gaining insight into the nature of the RNA conformational changes that occur on binding protein S4 to the S4-RNA, we employed two physical methods that are useful and complementary probes for detecting conformational alterations in RNA:

(i) Ultraviolet absorbance thermal denaturation, which yields insight into the thermal stability of the RNA structure in the absence and presence of the protein;

(ii) Circular dichroism, a sensitive probe of the secondary and probably tertiary structure of RNA (e.g., [7,8]).

No significant changes are seen in the circular dichroism spectrum of the S4-RNA as a result of binding to protein S4. However, in the presence of protein S4 the thermally induced unfolding of the S4-RNA shows a greatly enhanced cooperativity. We interpret these findings to indicate either that protein S4 induces an increase in conformational homogeneity of the S4-RNA population, or that it 'tunes' the helices of the S4-RNA such that they have similar conformational energies.

2. Materials and methods

2.1. *Preparation of the S4-RNA and protein S4*

16 S RNA was extracted by the phenol–dodecylsulphate method and renatured by heating in the presence of 20 mM Mg^{2+} and slowly cooling [9]. It was degraded with carrier-bound pancreatic ribonuclease, as in [4,5] and the S4-RNA was separated from the smaller degradation products chromato-

* Present address: Institute of Molecular Biology, University of Oregon, Eugene, OR 97403, USA

graphically [4]. Protein S4 was prepared in collaboration with Dr E. Schiltz using a modified version of the procedure in [10]. It was checked for identity by 2-D gel electrophoresis [11] and for purity by dodecylsulphate gel electrophoresis [12]; no contaminant proteins were detected. Protein concentrations were determined by the Lowry method [13].

2.2. *Gel electrophoresis*

The integrity of the S4-RNA, its purity, and the S4-RNA : S4–RNP ratio, were determined electrophoretically. S4-RNA (20 μg) or complex, were loaded onto a cylindrical 6% polyacrylamide gel containing TMK buffer (30 mM Tris–HCl (pH 7.6), 20 mM $MgCl_2$, 200 mM KCl). Electrophoresis was at 4°C with circulating buffer, at 12 mA/tube for 6 h. The gels were stained for RNA with pyronin G and for protein with Coomassie brilliant blue [14]. The S4-RNA migrated faster than the S4–RNP. The densities of the stained bands were measured with a Joyce-Loebl microdensitometer and the S4-RNA : S4–RNP ratio was estimated directly.

2.3. *Hypochromicity measurements*

In a typical experiment S4-RNA (100 μg) was dissolved in 0.2 ml HMK buffer (30 mM Hepes–KOH (pH 7.6), 20 mM $MgCl_2$ and 300 mM KCl). The S4–RNP was formed by adding a 1–3-fold molar excess of protein S4 and incubating for 30 min at 20°C, cooling slowly to 0°C, and diluting to 3 ml. Solutions were degassed by a gentle argon purge for 30 s. Ultraviolet A_{260} measurements were made on a Beckman Acta V spectrophotometer equipped with an Auto sampler accessory. Temperature was monitored by a platinum resistance thermometer inserted into a dummy cuvette. The cuvettes were sealed with General Electric RTV-88 silicon rubber to prevent evaporation. Absorbance temperature measurements were made every 25 s. The rate of heating was 0.02°C/s. Transition temperatures (T_m) were determined as outlined in [15] and from derivative melting curves.

2.4. *Circular dichroic spectra*

Circular dichroic spectra were recorded using a Durrum-Jasco J20 spectropolarimeter calibrated with camphorsulphonic acid d_{10} [16]. Solutions of S4-RNA were made up in sterile, Millipore-filtered HMK buffer at 20 μg/ml in 0.55 ml and spectra were recorded using a 1 cm pathlength cylindrical cell. A_{max} was ⊁ 1.2. The temperature of the sample block was controlled by a circulating water bath to ±1°C. Protein S4 displayed no significant A_{260} and no optical activity from 250–330 nm at the concentrations used in our experiments. At large molar protein : RNA ratios of ≳ 4:1, precipitation was observed. After clarifying the solutions by centrifugation a reduction in molar ellipticity was observed. This could not be completely accounted for by removal of precipitated RNA from solution and suggested some loss of RNA secondary structure. Larger reductions in ellipticity were observed upon adding S4 to a variety of single- and double-stranded polynucleotides [17]. The results suggest that some non-specific binding of S4 to the S4-RNA occurs at large molar protein excesses.

3. Results

The thermal denaturation profile of the S4-RNA, in HMK buffer, is shown in fig.1. After a slow initial melting stage between 32°C and 38°C, the S4-RNA melts gradually over a broad range of at least 40°C with a minimum of 24% hypochromicity (corrected for water expansion) and T_m ~58°C. This type of melting behavior is characteristic of non-cooperative unfolding, as exemplified by neutral polyadenylic acid [18]. When protein S4 was added to the S4-RNA under conditions favoring complex formation, the melting profile was markedly altered (see fig.1). There was a delay in the onset of melting from 32–42°C, indicating either:

(i) That the initial melting occurred on protein binding; or

(ii) That a portion of the RNA structure was stabilized by the protein.

More importantly, the RNA exhibited a significantly sharper transition from 42–62°C (T_m ~52°C) suggesting a large increase in the cooperativity of unfolding. The increase in cooperativity was dependent upon the amount of added protein, with the sharpest transition occurring when a 1:1 protein–RNA complex was formed (3-fold molar excess of protein). At all three protein–RNA ratios studied the T_m of the RNA was 52 ± 1°C. The dramatic increase in the cooperativity of RNA unfolding mediated by bound protein is also

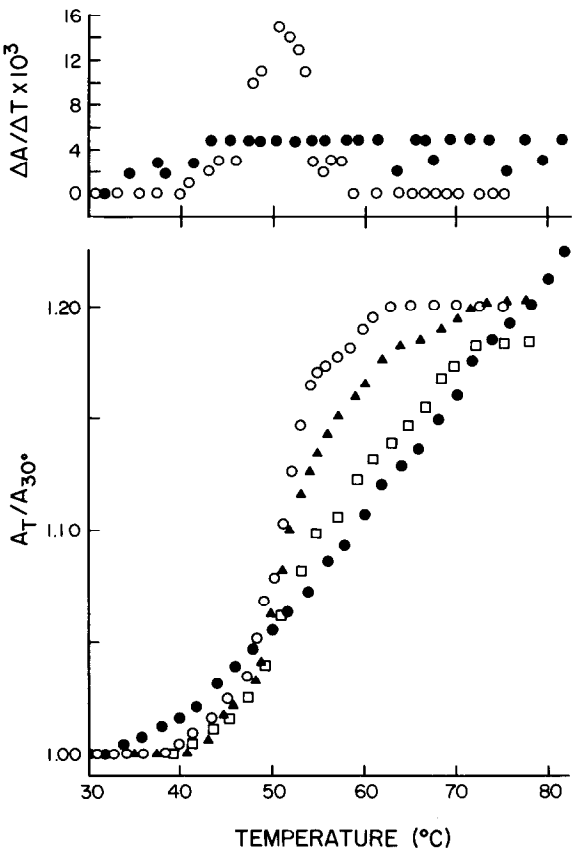

Fig.1. Ultraviolet thermal denaturation profiles for the S4-RNA (•) and the S4–RNP at 1:1 (□), 2:1 (▲) and 3:1 (○) protein : RNA ratios. Measurements were made as described in methods.

evident in the derivative melting curves ($\Delta A/\Delta T$ versus T) as shown in fig.1. At >59°C there was further non-cooperative melting resulting in approximately the same overall hypochromicity of 22% (corrected for water expansion). The hypochromicity values indicate a significant level of secondary structure, arising from base-stacking interactions, in both the free and complexed forms of the S4-RNA [15].

The thermal stabilities of the S4-RNA and the S4–RNP were examined electrophoretically. Samples of S4-RNA or S4–RNP were incubated for 10 min at a given temperature between 0°C and 60°C, rapidly cooled by freezing in liquid nitrogen, in order to minimize renaturation of the RNA structure, and electrophoresed in polyacrylamide gels, as in section 2.

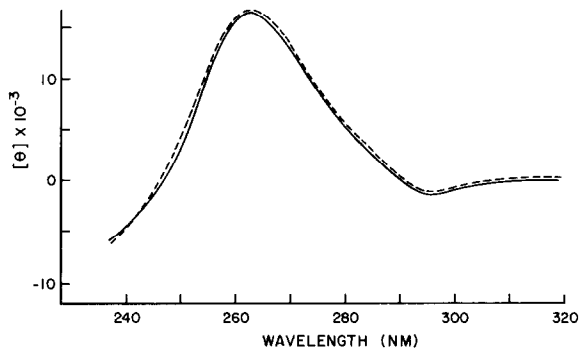

Fig.2. Circular dichroic spectra of the S4-RNA (—) and S4–RNP (- - -) determined at 21°C in HMK buffer.

The results (data not shown) confirmed that protein S4 stabilizes the S4-RNA against melting over the early part of the melting range shown in fig.1.

The magnitude of the RNA structural changes that occurred when protein S4 was complexed were investigated by circular dichroic spectroscopy. The spectra of the S4-RNA were measured in the absence and presence of protein S4. A spectrum of the S4-RNA (fig.2) differs from those of most other ribosomal RNA molecules (e.g., [7,8]) in that the circular dichroic band at 298 nm is exceptionally small. Addition of protein S4, stepwise, up to a 3:1 molar excess such that a 1:1 molar complex was formed, produced no detectable change in the spectrum (fig.2).

4. Discussion

Carrier-bound ribonuclease digestion [3] and low angle X-ray scattering studies [19] have demonstrated that the S4-RNA retains essentially the same structure when complexed with protein S4. It has been proposed, also, on the basis of ribonuclease degradation [20] and chemical modification studies on 30 S subunits [21], that this structure is essentially conserved in the 30 S subunit [4]. Nevertheless, there were indications that minor structural changes occur on binding protein S4. For example, some sites in the S4-RNA were slightly more accessible to carrier-bound pancreatic ribonuclease [3] and a few guanine residues were more reactive to kethoxal [6]. Given these results it was surprising that no change in the optical activity of the

S4-RNA was observed, on complexing protein S4. This indicates that the structural changes that occur are compensatory or produce only minor alterations in optical activity. This interpretation is compatible with the thermal melting results that are now considered.

The very different thermal melting behavior of the S4-RNA, the free form and in its complex with protein S4 (fig.1), shows that the S4 in some way influences this transition such that it becomes much more strongly cooperative. Since the melting of RNA helices is known to be cooperative [22], the non-cooperative melting of the S4-RNA (fig.1) is probably due to the summation of a number of cooperative transitions, constituting a heterogeneous range of thermal stability. When protein S4 is bound, this heterogeneity is largely eliminated. The effect can be interpreted in two formal ways:

1. In the first interpretation, the broad thermal transition is due to a heterogeneous population of S4-RNA conformers. The effect of protein S4 would then be to favor the formation of a single conformation (or thermally similar conformers).
2. The second interpretation is that the non-cooperative melting of the S4-RNA is due to the presence of a large number of helices (or other secondary and tertiary structures) within an individual S4-RNA molecule each of which have a broad range of thermal stabilities. In this case, the effect of S4 would be to 'tune' the helices of the S4-RNA in such a way that most of them have (or become part of the structures which have) similar conformational energies. Because of the existence of more stable, as well as less stable, structural elements in the S4-RNA as compared with the S4–RNP (fig.1), the effect of S4 must be to destabilize some structures as well as to increase the stability of others.

Our data do not allow us to distinguish between interpretations 1 and 2. The distinction between these two possibilities should yield further insight into the fundamental mechanism of the role of protein S4 in ribosomal assembly.

Acknowledgements

We wish to thank K. Katze for helpful discussions. This work was supported by grant no. GM-17129 (to H.F.N.) and GM-23951 (to T.S.) from the USPHS, and grants from the Deutsche Forschungsgemeinschaft (to R.A.G.).

References

[1] Mizushima, S. and Nomura, M. (1970) Nature 226, 1214–1218.
[2] Mackie, G. A. and Zimmermann, R. A. (1975) J. Biol. Chem. 250, 4100–4112.
[3] Ehresmann, C., Stiegler, P., Carbon, P., Ungewickell, E. and Garrett, R. A. (1977) FEBS Lett. 81, 188–192.
[4] Garrett, R. A., Ungewickell, E., Newberry, V., Hunter, J. and Wagner, R. (1977) Cell Biol. Int. Rep. 1, 487–502.
[5] Österberg, R., Sjöberg, B., Ungewickell, E. and Garrett, R. A. (1977) FEBS Lett. 80, 169–172.
[6] Noller, H. F. and Garrett, R. A. (1979) in preparation.
[7] Cox, R. A., Hirst, W., Godwin, E. and Kaiser, I. (1976) Biochem. J. 155, 279–295.
[8] Bear, D. G., Schleich, T., Noller, H. F. and Garrett, R. A. (1977) Nucl. Acids Res. 4, 2511–2526.
[9] Ungewickell, E., Garrett, R. A. and Le Bret, M. (1977) FEBS Lett. 84, 37–42.
[10] Hindennach, I., Stoffler, G. and Wittmann, H. G. (1971) Eur. J. Biochem. 23, 7–11.
[11] Kaltschmidt, E. and Wittmann, H. G. (1970) Anal. Biochem. 36, 401–412.
[12] Studier, F. W. (1973) J. Mol. Biol. 79, 237–248.
[13] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–276.
[14] Garrett, R. A., Rak, K. H., Daya, L. and Stoffler, G. (1971) Mol. Gen. Gen. 114, 112–124.
[15] Bloomfield, V. A., Crothers, D. M. and Tinoco, I., jr (1974) Physical Chemistry of Nucleic Acids, Harper and Row, New York.
[16] De Tar, D. F. (1969) Anal. Chem. 41, 1406–1408.
[17] Bear, D. G. (1978) PhD Thesis, University of California, Santa Cruz.
[18] Leng, M. and Felsenfeld, G. (1966) J. Mol. Biol. 15, 455–466.
[19] Österberg, R., Sjöberg, B., Garrett, R. A. and Littlechild, J. (1978) Nucl. Acids Res. 5, 3579–3587.
[20] Fellner, P., Ehresmann, C., Ebel, J. P. and Blasi, O. (1970) Eur. J. Biochem. 13, 583–588.
[21] Noller, H. F. (1974) Biochemistry 13, 4694–4703.
[22] Felsenfeld, G. and Miles, H. T. (1967) Ann. Rev. Biochem. 36 II, 407–448.